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Molecular characterization of three mitoviruses co-infecting a hypovirulent isolate of *Sclerotinia sclerotiorum* fungus

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ABSTRACT

Three double-stranded RNAs (dsRNAs) of 2438 nts (A), 2588 nts (B), and 2744 nts (C), from a single isolate of *Sclerotinia sclerotiorum* were sequenced. All three sequences showed similarity to known mitoviruses, consisting of a single open reading frame (ORF) with the characteristic conserved motifs of RNA-dependent RNA polymerase (RdRp). Mitochondrial malformations and reduced virulence and growth were associated with the presence of the dsRNAs. The terminal sequences of the (+) strand of the three dsRNAs could be folded into stem-loop structures and the inverted terminal complimentary sequences of dsRNA-A potentially form a panhandle structure. Sequence A showed 91.6% aa similarity to the previously described *Sclerotinia sclerotiorum* mitovirus 2 and was tentatively assigned the acronym SsMV2/NZ1. Sequences B and C showed only 16.4% similarity to each other and 15–48% aa similarity to the previously described mitoviruses and consequently appear to be new mitoviruses.

Introduction

Mycoviruses have been described in diverse fungal species including phytopathogens (Pearson et al., 2009). Although many cryptic or latent symptomless infections have been reported (Buck, 1998), the association of mycoviral infection with debilitated fungal phenotypes (Ghabrial and Suzuki, 2009; Pearson et al., 2009) has prompted mycovirologists to exploit mycoviruses as biological control agents against phytopathogenic fungi.

The simplest known mycoviruses are those assigned to the family Narnaviridae which includes two genera, Narnavirus and Mitovirus. Members of the genus Mitovirus replicate in strict association with their host mitochondria (Cole et al., 2000; Ghabrial and Suzuki, 2009). Mitoviruses have been reported in various plant pathogenic fungi including *Rhizoctonia solani* (Lakshman and Tavantzis, 1994; Lakshman et al., 1998), *Cryphonectria parasitica* (Polashock and Hillman, 1994), *Ophiostoma novo-ulmi* (Hong et al., 1999), *Sclerotinia homoeocarpa* (Deng et al., 2003), *Helicobasidium mompa* (Osaki et al., 2005), *Chalara elegans* (Park et al., 2006), *Botrytis cinerea* (Wu et al., 2007), *Sclerotinia sclerotiorum* (Xie and Ghabrial, 2012) and also in mychorrhizal fungi (Stielow et al., 2012).

The phytopathogenic fungus *S. sclerotiorum* is capable of causing significant yield losses in numerous crops in New Zealand and worldwide. It has a host range of over 400 plant species worldwide and is considered a serious threat to many economically important crops (Boland and Hall, 1994; Garg et al., 2010). Chemical disease management is largely unreliable as fungicide-resistant strains of the pathogen develop (Bolton et al., 2006; Li et al., 2008).

A range of mycoviruses have previously been found in *S. sclerotiorum* (Boland, 1992; Liu et al., 2009; Xie and Ghabrial, 2012; Xie et al., 2006,2011) including the only sequenced DNA mycovirus, *Sclerotinia sclerotiorum* hypovirulence-associated DNA virus 1 (Yu et al., 2010).

Results

Detection and sequencing of dsRNAs from S. sclerotiorum isolate 16235

Electrophoresis of dsRNA purified from the mycelium of *S. sclerotiorum* isolate 16235, yielded a slightly diffused band with a molecular weight of ~2.5 kb (Fig. 1A). Full-length sequence of 16235-dsRNA was obtained using random PCR and cloning with RACE-PCR to determine the 5′ and 3′ ends. Sequence assembly of 58 random cDNA clones revealed that the dsRNA band was composed of three distinct co-migrating dsRNA species (A, B and C). Further separation of 16235-dsRNA accentuated the presence of three distinct bands (Fig. 1A, Insert), confirming the sequencing results. BLAST searches of the full-length sequences of the three dsRNAs showed similarity to known mitoviral-RdRp sequences (Table 1). Sequence A showed 91.6% aa similarity to the previously described *S.*

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sclerotiorum mitovirus 2 (SsMV2/KL1) and was tentatively assigned the acronym SsMV2/NZ1. Sequences B and C showed only 15–48% similarity to previously described mitoviruses and were tentatively assigned the names *S. sclerotiorum* mitovirus 3 (SsMV3/NZ1) and *S. sclerotiorum* mitovirus 4 (SsMV4/NZ1), respectively. The relative abundance of the three mitoviruses was different with SsMV4/NZ1 being the most prominent. The complete nucleotide sequences of the three viruses were deposited in GenBank with accession numbers JX401536, JX401537 and JX401538, respectively.

Molecular characterization of the three mitoviruses

SsMV2/NZ1: The genome sequence of SsMV2/NZ1 consists of 2438 nucleotides (nts) with an A+U content of 55.1%. The lengths of the 5′- and 3′- untranslated regions (UTRs) of the positive (+) strand are 313 and 88 nt, respectively. Using the mitochondrial translation table, the genome consists of a single ORF of 2034 nts (nt positions 314 to 2350) which codes for a protein of 678 aa residues with expected molecular mass of 76.662 kDa. A BLASTP search of the protein showed 91.6% identity with RdRp of SsMV2/KL1. In contrast to SsMV2/KL1, SsMV2/NZ1 has no 3′ poly (A) but ends in four C's.

SsMV3/NZ1: The genome sequence of SsMV3/NZ1 has 2588 nts with a A+U content of 63.6%. Using the mitochondrial translation table, the genome consists of a single ORF of 2139 nts initiated by two adjacent AUG codons (AUGAUG) at nt position 298 and terminated at nt position 2436 by an UAG codon. The ORF is flanked by 5′- and 3′- UTRs of 297 and 152 nts, respectively, and has the potential to code for a protein of 712 aa residues with an expected molecular mass of 81.052 kDa. A BLASTP search of the protein showed 15.2% to 48.2% identity with RdRps of known mitoviruses (Table 1).

SsMV4/NZ1: The genome sequence of SsMV4/NZ1 is 2744 nts long and is rich in A+U content (69.7%). The lengths of the 5′- and 3′- UTRs of the (+) strand are 465 and 84 nts, respectively. Using the mitochondrial translation table, the genome consists of a single ORF of 2596 nts terminated by an opal codon. The ORF codes for a protein of 731 aa long with a calculated molecular weight of 85.608 kDa. A BLASTP search of the protein showed 15%–43.2% identity with RdRps of known mitoviruses (Table 1).

Fig. 1B shows a schematic representation of the genomic organization of the three mitoviruses. Alignments of the 5′- and 3′- UTRs of the three viruses showed no substantial stretches of identical or highly similar sequence. An alignment of the RdRp proteins of the three mitoviruses and 21 previously characterized mitoviruses shows the typical amino acid sequence motifs (I–IV) of RdRp, including the highly conserved GDD region in motif IV.

Predicted secondary structures of the mitoviruses 5′- and 3′- UTRs

The predicted secondary structures of the 5′- and 3′- UTRs of the three mitoviruses indicate the presence of terminal stem-loop structures that stabilize the RNA genomes. The 5′ (nt positions 1–30) and 3′ (nt positions 2367–2438) terminal sequences of SsMV2/NZ1 (+) RNA could be folded into stem-loop structures with ΔG values of −14.1 and −27.4 kcal/mole, respectively (Fig. 2A, B). Moreover, a cruciform-shaped panhandle structure was formed by inverted terminal complimentary sequences at both 5′ and 3′ end (Fig. 2C). The (+) strand of SsMV4/NZ1 could be folded at 5′- (nts 1–47) and 3′- (nts 2699–2744) UTRs to form stem-loop structures with ΔG values of -19.5 and −22.2 kcal/mole, respectively (Fig. 2D, E). Stem-loops with ΔG values of −16.4 and −37.3 kcal/mole were predicted at sequence positions 1–60 and 2489–2588 of the 5′- and 3′- UTRs of SsMV3/NZ1, respectively (Fig. 2F, G). Unlike SsMV2/NZ1, the 5′- and 3′-UTRs of SsMV3/NZ1 and SsMV4/NZ1 could not be folded into stable panhandle secondary structure.

Interviral relationships of the three mitoviruses

Phylogenetic analysis of the RdRp proteins of SsMV2/NZ1, SsMV3/NZ1 and SsMV4/NZ1 and other mitoviruses (Fig. 3) divides

Table 1
Percent aa sequence identity (RdRp) between SsMV2/NZ1, SsMV3/NZ1, SsMV4/NZ1 and other members of the genus Mitovirus.

Mitovirus	Acronym	RdRp			Presence of poly (A)	GenBank accession no.
		SsMV2/NZ1	SsMV3/NZ1	SsMV4/NZ1		
Botrytis cinerea mitovirus 1	BcMV1	15.3	32.5	17.5		ABQ65153
Cryphonectria cubensis mitovirus 1a	CcMV1a	24	17.8	19.6	√	AAR01970
Cryphonectria cubensis mitovirus 2a	CcMV2a	15.2	24.2	15.9		AAR01973
Cryphonectria parasitica mitovirus 1-NB631	CpMV1-NB631	14.7	21.5	15.4		NP_660174
Gremmeniella abietina mitochondrial RNA virus S2	GaRV-MS2	21	16.6	31.3		YP_077184
Gremmeniella mitovirus S1	GMV-S1	21.1	16.5	31.2		AAN05635
Helicobasidium mompa mitovirus 1-18	HmMV1-18	19.1	16.9	21.8		BAD72871
Ophiostoma mitovirus 1a	OMV1a	16.1	20.9	16.6		CAJ32466
Ophiostoma mitovirus 1b	OMV1b	14.8	20.7	15		CAJ32467
Ophiostoma mitovirus 3a	OMV3a	15.9	48.2	15.9		NP_660176
Ophiostoma mitovirus 3b	OMV3b	15.6	33	17.8		CAJ32468
Ophiostoma mitovirus 4	OMV4	19.6	18.4	43.2		NP_660179
Ophiostoma mitovirus 5	OMV5	22.3	17.3	33.9		NP_660180
Ophiostoma mitovirus 6	OMV6	20.6	18.9	26		NP_660181
Sclerotinia homoeocarpa mitovirus	ShMV	15.6	48	16.2		AAO21337
Sclerotinia sclerotiorum mitovirus 1/KL1	SsMV1/KL1	22.7	19.6	37.4		AEX91878
Sclerotinia sclerotiorum mitovirus 2/KL1	SsMV2/KL1	91.6	15.3	20.4	√	AEX91879
Sclerotinia sclerotiorum mitovirus 2/NZ1	SsMV2/NZ1		15.2	20.6		JX401536[a] JX401537[a]
Sclerotinia sclerotiorum mitovirus 3/NZ1	SsMV3/NZ1	15.2		16.4		JX401538[a]
Sclerotinia sclerotiorum mitovirus 4/NZ1	SsMV4/NZ1	20.6	16.4			
Tuber aestivum mitovirus	TaMV	14.9	19	15.5		YP_004564622
Thielaviopsis basicola mitovirus	TbMV	22.9	18.1	37.4		YP_002822229
Thanatephorus cucumeris mitovirus	TcMV	16.2	20.9	15.8		AAD17381
Tuber excavatum mitovirus	TeMV	14.7	15.3	16.8		AEP83726

[a] Nucleotide sequence accession numbers.

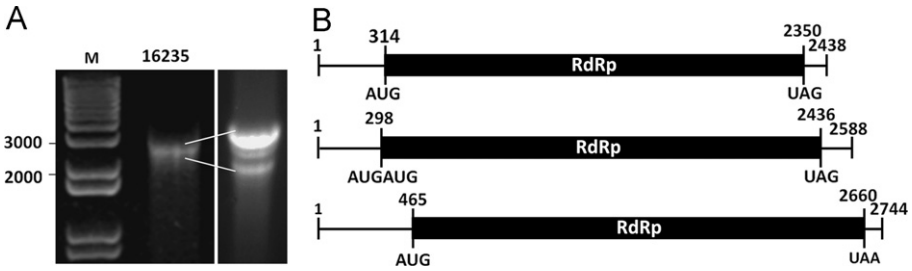

Fig. 1. (A): Agarose gel electrophoresis of dsRNA associated with isolate 16235 (1% gel, 1 h running time). M: 1 kb plus marker. (Insert: 1.5% gel, 5 h running time). (B): Schematic representation of SsMV2/NZ1 (2438 nts), SsMV3/NZ1 (2588 nts), and SsMV4/NZ1 (2744 nts) genomes. (+) strand of each mitovirus genome contains a long single ORF encoding RdRp protein and 5′- and 3′-UTRs of varying size.

mitoviruses into two clades with SsMV2/NZ1and SsMV4/NZ1 in clade I and SsMV3/NZ1 in clade II. Pairwise aa identities between SsMV2/NZ1, SsMV3/NZ1 and SsMV4/NZ1 and previously identified mitoviruses are presented in Table 1. The highest aa identity (91.6%) is between SsMV2/NZ1and SsMV2/KL1, while the highest identities for SsMV3/NZ1and SsMV4/NZ1 were 48.2% (Ophiostoma mitovirus 3a) and 43.2% (Ophiostoma mitovirus 4 (OMV4)), respectively.

Horizontal virus transmission and associated hypovirulence

The three mitoviruses were concurrently transmitted from the originally infected isolate 16235 to isolate 13844sh^{hyg} by dual culture, the resultant infected isolate being labeled 13844sh^{hyg}+V. The presence of the three viruses in 13844sh^{hyg}+V was verified by RT-PCR (Fig. 4C). The virus transfected isolate 13844sh^{hyg}+V showed slower growth on PDA plates compared to the parental isolate 13844sh^{hyg} (Figs. 4 and 5A). The in-vitro growth rate of the naturally infected isolate 16235 was 1.56 cm/d compared to 2.11 cm/d for the uninfected isolate 13844sh^{hyg} (Figs. 4 and 5A). When isolate 13844sh^{hyg} was infected with the dsRNAs of isolate 16235 via hyphal anastomosis, its growth rate reduced to 1.75 cm/d, similar to that of 16235. The virus infected isolates also exhibited reduced virulence on detached leaves of oilseed rape, common bean, tomato, and cabbage (Fig. 5B E). Fig. 4B shows a comparison of lesion diameters developed by the virus- free and virus-containing isolates on tomato leaves as a representative host for virulence assessment. Isolates 16235 and 13844sh^{hyg}+V showed smaller lesion diameters of 0.91 cm and 1.25 cm, respectively, compared to a lesion of 2 cm in diameter developed by isolate 13844sh^{hyg}.

Relative contribution of each of the three mitoviruses to fungal hypovirulence

Attempts, using chemical treatment with cycloheximide and incubation at high temperatures, to eliminate one or more of the three mitoviruses failed (Supplementary Fig. S1). Incubation at 28 °C of colonies treated with 20 and 25 μg/ml cycloheximide was growth inhibitory.

100 ascospore progeny were collected and screened for the presence of mitoviruses using dsRNA detection and RT-PCR. None of the isolates were found to be cured of all three mitoviruses. SsMV2/NZ1 was found to be transmitted to all progeny whereas SsMV3/NZ1 and SsMV4/NZ1 were found to be transmitted randomly, in different combinations, to 13% and 87% of the progeny, respectively. As shown in Fig. 6A, based on the combination of the mitoviruses present, ascospore progeny were separated into three groups. Progeny of group I (represented by isolate 16235-sa2) harbor SsMV2/NZ1. Group II (represented by isolate 16235-sa9) contains progeny with the three mitoviruses whereas group III progeny (represented by isolate 16235-sa11) contain SsMV2/NZ and SsMV4/NZ1. The reduction in virulence of the progeny in groups I and III was not significant. Progeny of group II showed slightly reduced growth rate (Fig. 6B) and virulence (Fig. 6C, D) as compared to that of isolate 13844sh^{hyg} (virus free) and similar to that of isolate 16235 on tomato detached leaves.

Ultrastructural changes of mitochondria

Electron microscopic observation of ultrathin sections of the hyphae of isolate 16235 showed swollen and malformed mitochondria with destruction or loss of cristae (Supplementary Fig. S2). The virus uninfected isolate 13844sh^{hyg} showed normal cellular organelles.

Discussion

Viruses of the genus Mitovirus (family Narnaviridae) are the simplest unencapsidated mycoviruses known to date. They use their host mitochondrial translation machinery for protein synthesis and have been co-purified with their host mitochondria (Cole et al., 2000). Mitoviruses typically have RNA genomes of 2.3–2.7 kb with ~62–73% A+U content, consist of a single ORF which codes for an RdRp protein and their 5′ and 3′ sequences are able to form stable stem-loop structures (King et al., 2012). However, the recently described SsMV2/KL1 differs slightly in having a 53.1% A+U content and a poly (A) tail at the 3′ end (Xie and Ghabrial, 2012). Two of the three viruses characterized in this current study have characteristics of typical mitoviruses while the third is very similar to SsMV2/KL1. The most notable difference between is that SsMV2/KL1 has a poly (A) tail at its 3′ end whereas SsMV2/NZ1, in common with most mitoviruses, does not.

The genome organizations of the three mitoviruses are similar (Fig. 1B), having a single ORF with typical aa sequence motifs of RdRps and similar to RdRp motifs described for other mitoviruses. In fungal mitochondria, UAA is the preferred, and sometimes the only, termination codon (Paquin et al., 1997), which is the case for SsMV4/NZ1. However, in SsMV2/NZ1, SsMV3/NZ1 and seven previously identified mitoviruses, UAG is used as a translation stop codon. SsMV3/NZ1 and SsMV4/NZ1 share the common character of fungal and plant mitochondrial genomes in having A+U-rich genome content (Hong et al., 1998a) (SsMV3/NZ1=63.6%; SsMV4/NZ1=69.7%). SsMV2/NZ1 genome has a relatively low A+U content (55.1%) similar to that of SsMV2/KL1 (53.1%) and *Cryphonectria cubensis* mitovirus 1a (50.5%).

ORFs of SsMV3/NZ1and SsMV4/NZ1 have a codon preference of A or U in the third position (72% and 82%, respectively) which is characteristic of mitochondrial codons (Paquin et al., 1997), while SsMV2/NZ1 has a lower frequency (54.5%) which is similar to SsMV2/KL1 (50%).

Another characteristic feature of fungal mitochondrial viruses is the potential of the 5′ and 3′ terminal sequences of the (+) strand

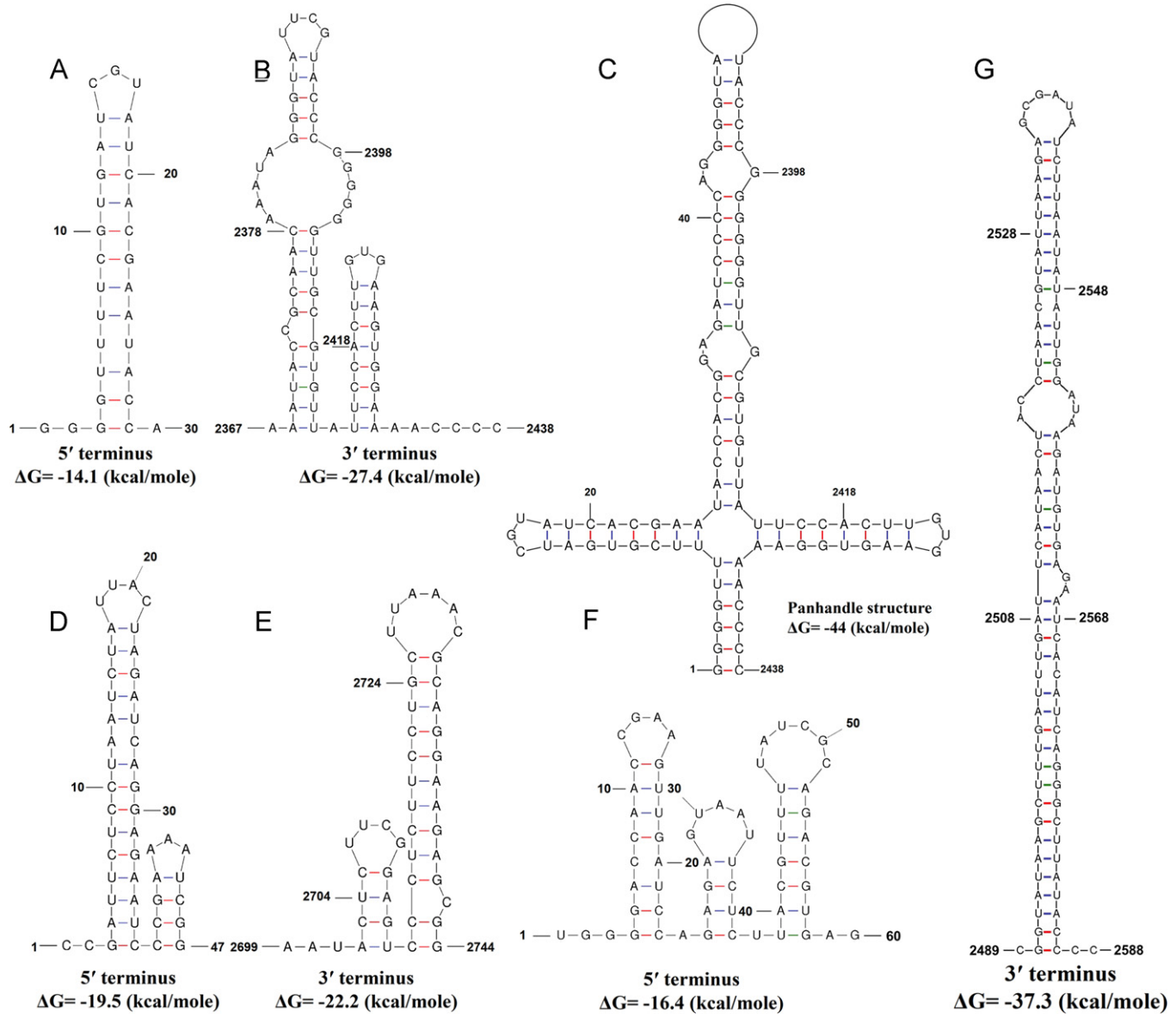

Fig. 2. Potential predicted secondary structures of the 5′- and 3′-UTRs of the (+) strand of SsMV2/NZ1 (A, B), SsMV4/NZ1 (D, E) and SsMV3/NZ1 (F, G). Panhandle structure formed by complementary sequences of the 5′- and 3′- terminal sequences of SsMV2/NZ1 (C). The RNAs were folded and the free energy (ΔG) was estimated using MFOLD software (Mathews et al., 1999).

to form stem-loop and hairpin structures (Hong et al., 1998b). The terminal sequences might be inverted complimentary repeats and fold into panhandle structures (Hong et al., 1998b). These structures may play an important role in mitoviral replication, acting as recognition sites for the RdRp (Buck, 1996; Buck and Brasier, 2002). Analysis of the termini of all three viruses indicates they could potentially be folded into stable stem-loop structures (Fig. 2). In addition, the inverted complementarity nature of the 5′ and 3′ termini would allow the (+) strand of SsMV2/NZ1 to form panhandle (cruciform) structure (Fig. 2C). Panhandle structures were previously identified in several mitoviruses including OMV4, Ophiostoma mitovirus 6 (Hong et al., 1999), *Helicobasidium mompa* mitovirus 1–18 (Osaki et al., 2005) and *Thielaviopsis basicola* mitovirus (TbMV) (Park et al., 2006).

The three viruses were found in a single *S. sclerotiorum* isolate (16235). Infection of a single fungal isolate by multiple virus-like elements is not unusual and has been reported in several fungal species such as *B. cinerea* (Howitt et al., 2006), *Helminthosporium victoriae* (Ghabrial et al., 2002), *O. novo ulmi* (Cole et al., 1998), *Rosellinia necatrix* (Sasaki et al., 2005), and *S. sclerotiorum* (Xie and Ghabrial, 2012). Low aa sequence identities (15.2–20.6%) between pairwise combinations of the three mitoviruses and the ability of each mitoviral genome to encode RdRp suggest the unrelatedness and the independent replication of the three mitoviruses.

Phylogenetic analysis of the complete genome sequences indicated that SsMV2/NZ1 (clade 1) had 91.6% aa identity with SsMV2/KL1 and consequently should be considered an isolate of SsMV2. SsMV3/NZ1 (clade 2) and SsMV4/NZ1 (clade 1) showed only 16.4% similarity to each other and 15–48% aa similarity to previously described mitoviruses. Therefore they appear to be new mitoviruses. However it is noteworthy that nt positions 825–1446 of SsMV4/NZ1 has 96.5% identity with a 622 nt partial sequence (GenBank accession no. GU108590) obtained by pyrosequencing of dsRNAs extracted from a grapevine sample in the US (Al Rwahnih et al., 2011). This sequence was identified as a partial sequence of grapevine associated narnavirus-1 isolate Ctg501. If these are

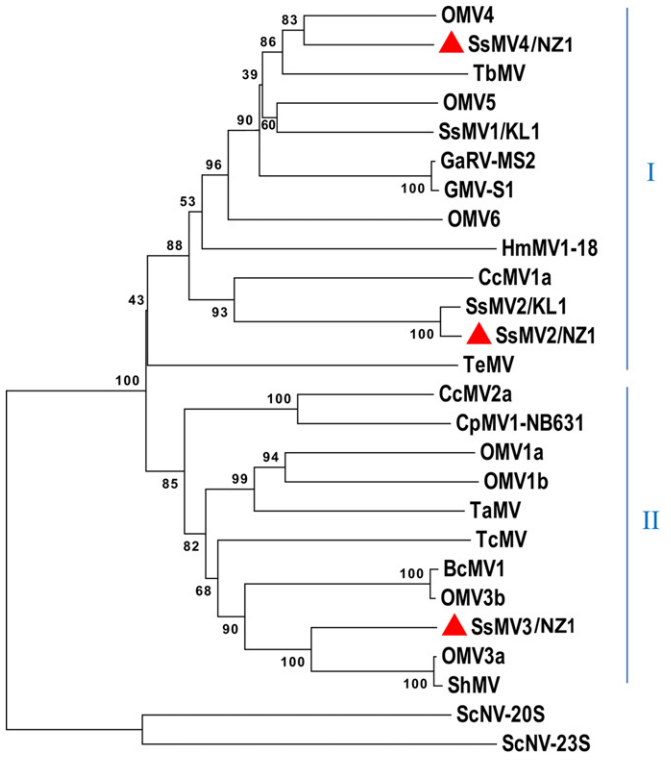

Fig. 3. Evolutionary relationship between SsMV2/NZ1, SsMV3/NZ1, SsMV4/NZ1, 22 members of the genus Mitovirus and two members of Narnavirus. Multiple sequence alignment of the deduced amino acid sequences of RdRp genes was conducted using CLUSTAL W software. The tree was displayed using MEGA 5 software. The results of bootstrapping analysis of 1000 replicates are indicated by numbers on the branches. Virus notations are as shown in Table 1. ScNV-20S, *Saccharomyces cervisiae* 20S narnavirus; ScNV-23S, *S. cervisiae* 20S narnavirus.

isolates of the same virus it is likely that Ctg501 is from an endophetic or phytopathogenic fungus associated with the grapevine stem sample. Al Rwahnih et al. (2011) reported that 11 fungal species were cultured from the same vine stem material, although *S. sclerotiorum* was not one of them and the Ctg501 sequence was not detected in any of them.

Mitoviral infection is often associated with reduced virulence of phytopathogenic fungi, such as *B. cinerea* (Wu et al., 2007), *C. parasitica* (Polashock and Hillman, 1994), *O. novo-ulmi* (Hong et al., 1999), *R. solani* (Lakshman and Tavantzis, 1994), *S. homoeocarpa* (Deng et al., 2003), and *S. sclerotiorum* (Xie and Ghabrial, 2012). However, the level of hypovirulence associated with mitovirus infection is variable. For example, *Cryphonectria cubensis* mitovirus 1b and *Cryphonectria cubensis* mitovirus 2a had little or no significant impact on their host (Van Heerden, 2004) while B. cinerea was severely debilitated when infected by *Botrytis cinerea* mitovirus 1 (BcMV1) (Wu et al., 2007). Our results showed that the three mitoviruses, in combination, significantly reduced both the in-vitro growth (Fig. 4A) and virulence of *S. sclerotiorum* on four different hosts (Fig. 5B–E). Although there was a general and statistically significant effect on growth and pathogenicity, there was some variability between experimental replicates and different subcultures. This was possibly the result of uneven distribution and movement of the different mitoviruses within the growing mycelium.

Polashock et al. (1997) shed the light on the importance of mitochondrial dynamics (fusion and fission) in the spread and transmission of *Cryphonectria parasitica* mitovirus 1 (CpMV1). They reported that mitochondrial movement and recombination of the mitochondrial genome were associated with the transmission of CpMV1 via hyphal anastomosis. Our results showed that the three mitoviruses could be transmitted via hyphal anastomosis which might be similarly associated with mitochondrial movement and fusion.

Conidiogensis of *O. novo-ulmi* led to the loss of different combinations of dsRNA elements (Cole et al., 1998) and it was suggested that it could be due to concentrations of host proteins likely to be needed for RNA replication becoming limited (Buck, 1996). Similarly, sexual sporulation of *S. sclerotiorum* led to similar results although SsMV2/NZ1 was present in all progeny. Our attempts to determine the relative contribution of the individual viruses confirmed that the three mitoviruses in combination reduced the in-vitro growth and virulence of the single-ascospore progeny 16235-sa9 (Fig. 6B–D). Absence of SsMV3/NZ1 in single-ascospore progeny of isolate 16235 showed the wildtype phenotype of *S. sclerotiorum*. This finding suggests that SsMV3/NZ1 could be the hypovirulence determinant in isolates 16235 and 16235-sa9. However, it is not clear whether there is an interaction between the three mitoviruses to express the reduced virulence in *S. sclerotiorum*. Although SsMV4/NZ1 was present in the highest concentration compared to SsMV2/NZ1 and SsMV3/NZ1 (Fig. 1, Inset and 6A), it did not appear to play a significant role in the expression of the hypovirulent phenotype.

Ultrastructural mitochondrial malformations have previously been reported in mitovirus infected fungi, including smaller and fewer mitochondria in *C. elegans* infected with TbMV (Park et al., 2006) and malformed mitochondria with remnants of degenerated cristae and fibrous matrix materials, believed to be the viral RNAs, in *B. cinerea* infected with BcMV1 (Wu et al., 2010). Similarly, in the present study, the mitovirus free isolate 13844sh^{hyg} contained abundant and normal mitochondria (Supplementary Fig. S2), whereas the mitovirus infected isolate 16235 appeared vacuolated with swollen and severely disintegrated mitochondria that have unusual electron density and a few cristae remnants inside a degenerated matrix (Supplementary Fig. S2).

In summary, we have identified three mitovirus associated with a hypovirulent phenotype in *S. sclerotiorum*. One is an isolate of the previously described SsMV2/KL1 while the other two appear to be new viruses which we tentatively named SsMV3 and SsMV4.

Materials and methods

Fungal isolates

Fungal isolates were obtained from the International Collection of Microorganisms from Plants (ICMP). *S. sclerotiorum* ICMP# 16235 (isolate NZ1) was isolated in 2005 from infected *Petroselinum crispum* (wild parsley) stem in Mt Albert, Auckland, New Zealand; ICMP# 13844 was isolated in 1995 from an infected *Actinidia deliciosa* (Kiwifruit) fruit in Bethleheim, Bay of Plenty, New Zealand; isolate 13844sh is a single hyphal tip isolate of ICMP# 13844 and a sub-isolate was transformed with a hygromycin (^{hyg}) resistant gene as described by Rollins (2003). During the study all isolates were maintained on PDA plates. Isolates 16235-sa2, 16235-sa9 and 16235-sa11 are single-ascospore derivatives originating from isolate 16235.

Extraction and purification of dsRNA

DsRNA was extracted based on the selective binding to CF11 cellulose in the presence of ethanol as described by Valverde et al. (1990) and separated by electrophoresis in 1% (w/v) agarose gel in TAE buffer. The gel was pre-stained with RedSafe (iNtRON, Korea) and visualized and photographed under UV using a Gel Doc (Bio-Rad). The presence of dsRNA was confirmed by treating the

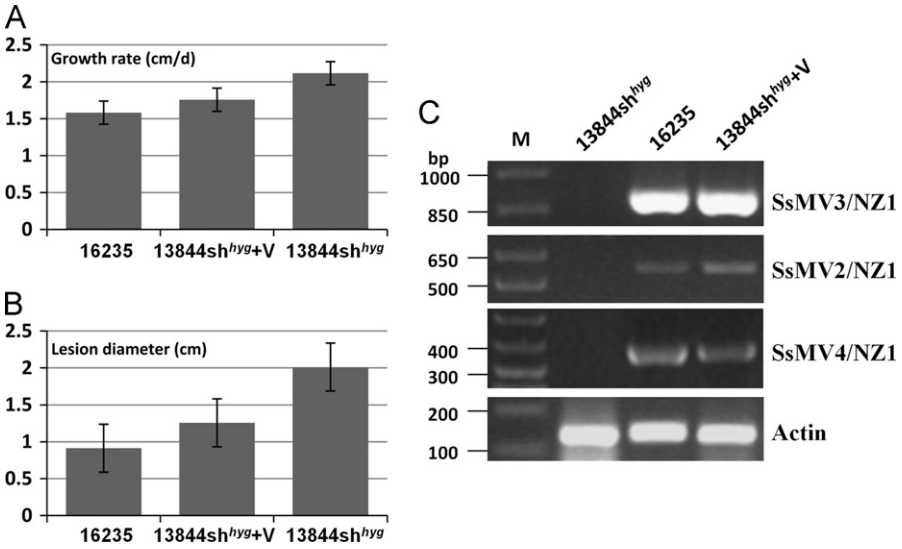

Fig. 4. Growth rate (A) and lesion diameter (B) comparisons between the mitovirus-harboring isolate (16235), the paired isolate (13844sh^{hyg}+V) and the mitovirus-free isolate (13844sh^{hyg}). The fungus was grown on PDA medium for 4-5 days at 20 °C. Growth rate was calculated using the equation: fungal growth radius (cm)/ incubation period (d). Lesion diameter measurements were taken after a 3 day incubation period of mycelium plugs on tomato leaves. (C). Amplified fragments by RT-PCR detection of SsMV2/NZ1 (609 bp), SsMV3/NZ1 (882 bp), SsMV4/NZ1 (341 bp) and actin gene of *S. sclerotiorum* (internal control) in the mitovirus-free isolate (13844sh^{hyg}), mitovirus-harboring isolate (16235) and the paired isolate (13844sh^{hyg}+V). Growth rates of isolate 16235 and isolate 13844sh^{hyg}+V were significantly different (P<0.050) from that of isolate 13844sh^{hyg}.

extracts with RQ1 DNase (Promega) and RNase A (Sigma) in both high and low salt conditions as described by Howitt et al. (1995). Nucleic acid molecules that were digested by RNase A in low salt buffer and resisted both DNase and RNase digestion in high salt buffer were considered dsRNA.

cDNA synthesis, PCR and sequencing

DsRNA from ICMP# 16235 was gel extracted and used for cDNA synthesis using a protocol based on that of Roossinck et al. (2010). A 3 μl aliquot of dsRNA was mixed with 5 μl H2O, 1 μl (10 mM) TE buffer and 1 μl (20 μM) the random primer 5′- CCTTCGGATCCTCC N10-12 -3′. The dsRNA was denatured by boiling for 2 min, quenched on ice and 8 μl of a reverse transcription mixture (1 μl Superscript III "Invitrogen", 4 μl 5X first strand buffer, 2 μl dithiothreitol and 1 μl (10 mM) dNTPs) added. The mixture was incubated at 50 °C for 40 min, treated with ribonuclease A (5 μg) and the remaining primers removed using a QIAquick PCR purification kit (Qiagen, USA), as described by the manufacturer.

A 5 μl aliquot of the cDNA was amplified in a reaction volume of 50 μl containing 10 mM Tris–HCl, pH 8.3; 3 mM MgCl2; 0.4 mM dNTP mix; 1 μM primer 5′-TCGTCCTTCGGATCCTCC-3′; 1.2M Betaine solution; and 1 unit of AmpliTaq DNA Polymerase. The amplification program was: 94 °C for 1 min; 65 °C 30 s; 72 °C 3 min, followed by 40 cycles of 94 °C 30 s; 45 °C 30 s; 72 °C 2 min, and a final 5 min at 72 °C and 5 min at 37 °C. PCR products (fragments with variable sizes) were either purified using a Qiagen PCR purification kit or resolved on 1% agarose gel followed by gel extraction using an AxyPrep DNA gel extraction kit (Axygen, USA). The PCR product was then cloned into pGEM-T easy vector (Promega, USA) and transformed into Escherichia coli DH5α. Subsequent clones were screened and sequenced using T7 and SP6 universal primers.

The terminal sequences were determined by ligating adapter T4L (5′-PO4-CCCGTCGTTTGCTGGCTCTTT-NH2-3′) to the 3′ end of the dsRNAs using T4 RNA ligase (Promega, USA). T4L-ligated dsRNAs were denatured either by boiling in 100% DMSO for 2 min and then quickly chilled on ice or by incubation in 25 mM methylmercury hydroxide at room temperature for 20 min. The denatured T4L-ligated dsRNAs were used for the reverse transcription reaction with Superscript III reverse transcriptase (Invitrogen) using T4LC primer (5′-AAAGAGCCAGCAAACGACGGG-3′) that has sequence complementary to the sequence of T4L adapter. Aliquots of the cDNA were used for PCR amplification with T4LC reverse primer and sequence-specific forward primers corresponding to dsRNAs A, B and C. PCR products were separated on 1% (w/v) agarose gel in TAE, gel-extracted using AxyPrep DNA gel extraction kit, cloned into pGEM-T easy vector and sequenced using T7 and SP6 universal primers.

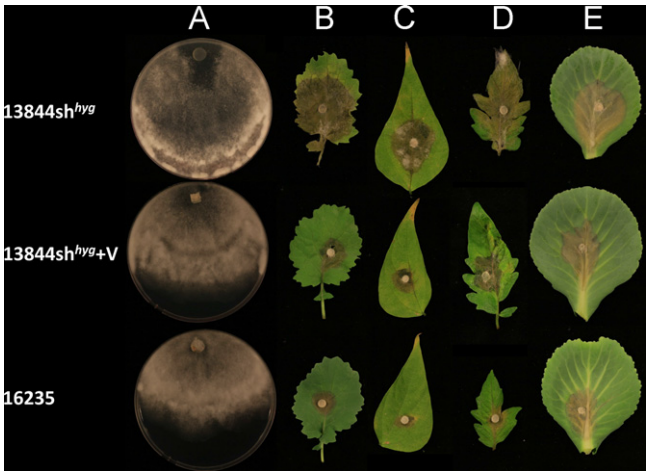

Fig. 5. Colony morphology (A) and pathogenicity assays of the mitovirus-free isolate (13844sh^{hyg}), the paired isolate (13844sh^{hyg}+V), and mitovirus-harboring isolate (16235) on oilseed rape (B), common bean (C), tomato (D) and cabbage (E) detached leaves. Photographs of colony morphology were taken after the inoculated PDA plates have been incubated for 5 days. Virulence on detached leaves was assessed 72 h post-inoculation.

Sequence analysis and phylogeny

Nucleotide sequences were assembled using Geneious version 5.6.5 (Drummond et al., 2011). Potential secondary structures at the 3′ and 5′ termini were predicted and the free energy (ΔG) was estimated using MFOLD software (http://mfold.rna.albany.edu/)

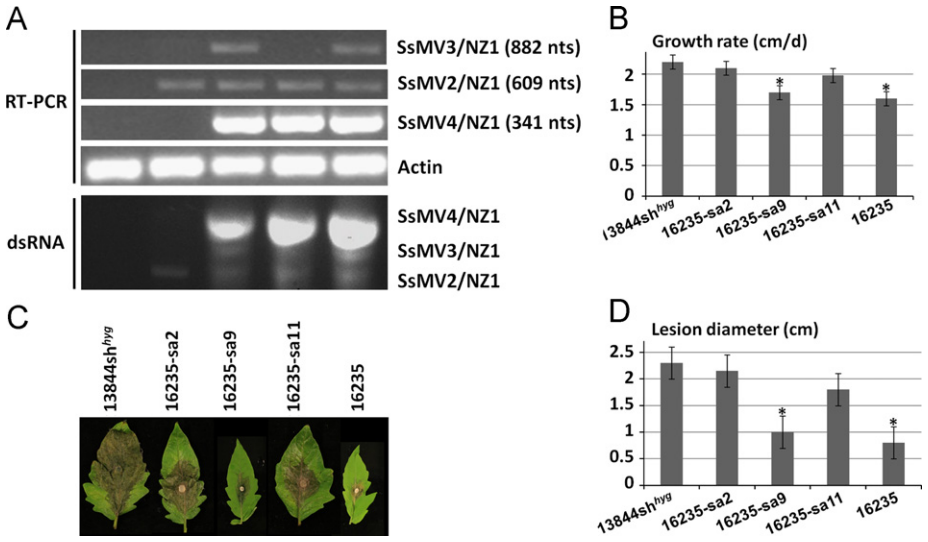

Fig. 6. (A): Detection of SsMV2/NZ1, SsMV3/NZ1 and SsMV4/NZ1 using dsRNA extraction and RT-PCR. The actin gene of *S. sclerotiorum* was used as internal control. (B): Growth rate comparisons between isolates 13844sh^{hyg}, 16235 and the single-ascospore derivatives of isolate 16235. (C, D): Lesion diameter comparisons between isolates 13844sh^{hyg}, 16235 and the single-ascospore progeny of isolate 16235, developed on detached leaves of tomato. Asterisks (*) indicate that values are significantly different (P<0.050) from that of isolate 13844sh^{hyg}.

(Mathews et al., 1999). For phylogenetic analysis, the tree construction and multiple sequence alignment of the deduced amino acid sequences of the RdRp genes were conducted using CLUSTALW plug-in of MEGA 5 (Tamura et al., 2011). Multiple sequence alignment for detection of conserved motifs was carried out using CLUSTAL OMEGA (Sievers et al., 2011).

Horizontal transmission of dsRNA

If the hypovirulent phenotype is caused by the presence of the mitoviruses it should be transmissible to a virulent fungal isolate via hyphal fusion (anastomosis) (Shain and Miller, 1992). Horizontal transmission of the dsRNAs from isolate 16235 to isolate 13844sh^{hyg} was conducted using the paired culture technique. Agar plugs of the donor and recipient isolates were placed 2 cm apart at one side on PDA plates and incubated at 20 °C to allow colony development. Following contact between the leading edges of the two colonies agar plugs were taken from the colony margin of isolate 13844sh^{hyg} and transferred onto PDA plates containing 100 μg/ml hygromycin. Agar plugs from the margin of the newly developed colony (13844sh^{hyg}+V) were subcultured on PDA prior to testing for the presence of dsRNA and assessing its growth rate and virulence.

Growth rate and virulence assay

PDA plates (three replicates) were inoculated at the margin with plugs from cultures of *S. sclerotiorum* isolates 13844sh^{hyg}, 13844sh^{hyg}+V and 16235 and radial growth measured after incubation for 5 days at 20 °C. Virulence was assessed on detached leaves of four susceptible hosts; oilseed rape, common bean, tomato and cabbage (eight replicates) placed in tissue culture tubs containing water agar (to maintain high humidity). The leaves were inoculated with 5 mm plugs of the fungal cultures and incubated for 3 days at 20 °C.

Transmission electron microscopy

Isolates 13844sh^{hyg} and 16235 were grown on a cellophane membrane placed on PDA. Small pieces of mycelium from the margins of the colonies were fixed in 2.5% (w/v) glutaraldehyde in 0.1 M Sörensens phosphate buffer (pH 7) for 3 h at 4 °C. Following fixation they were washed three times (10 min per wash) in 0.1 M Sörensens phosphate buffer (pH 7), post-fixed for 1 h in 1% (w/v) osmium tetroxide in 0.1 M Sörensens phosphate buffer, dehydrated in an ethanol series (30%, 50%, 70%, 90%, 1X and 100%, 2X) followed by 100% of acetone (2X, 10 min). Samples were infiltrated in a (1:1) mixture of 812 epoxy resin and acetone for 1 h followed by an infiltration step overnight in 100% 812 epoxy resin. Samples were transferred to embedding molds containing fresh resin and cured at 60 °C for 48 h. Ultra-thin sections (approximately 70 nm thick) were cut using a Leica EM UC6 ultramicrotome, picked up on copper 200 mesh grids, stained with 2% uranyl acetate and Reynold's lead citrate and examined using a Philips CM12 transmission electron microscope.

Attempts to eliminate the mitoviruses

Two approaches were used to eliminate one or more of the mitoviruses in isolate 16235. The first approach involved chemical and physical treatment. Hyphal tips from isolate 16235 were transferred onto PDA amended with cycloheximide (Sigma, USA) (15, 20 and 25 μg/ml). Three replicates of each concentration were incubated at temperatures 20, 25 and 28 °C for 7 days. The second approach used was single ascospore isolation. Carpogenic germination of sclerotia was carried out as described by Huang and Kozub (1991) with modifications. Sclerotia were harvested from cultures grown in the dark on PDA at 20 °C for 4 weeks, conditioned at 4 °C for 8–12 weeks and then germinated at 20 °C under daylight for 4 weeks. Hyphal tips from colonies developed by the first approach and ascospore progeny produced by the second approach were taken and subcultured on PDA plates at 20 °C prior to total RNA extraction and RT-PCR detection of the three mitoviruses as described below. Virulence was assessed on detached leaves of tomato, a representative host for virulence assay, as described previously.

RT-PCR detection of the three mitoviruses

For routine detection of the three dsRNAs, RNA was extracted from 100 mg of fungal mycelium using the Spectrum Plant Total RNA Kit (Sigma, USA) and RT-PCR conducted using virus specific primers (Table 1) based on the previously obtained sequence. As an internal control to confirm the PCR competence of the DNA, the

primers actin-qF2 (5′-GAGCTGTTTTCCC TTCCATTGTC-3′) and actin-qR4 (5′-GACGACACCGTGCTCGATTGG-3′) were used to amplify a 146bp-long fragment of the *S. sclerotiorum* actin gene (Sexton et al., 2009).

Statistical analysis

Growth rate and lesion diameter data were analyzed by one-way analysis of variance (ANOVA) using SigmaPlot 12.0 (SYSTAT Software). Differences with $P<0.05$ were considered statistically significant.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.03.002.

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